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Localization and Purification of Serum Albumin in the Testis of *Xenopus laevis*

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ABSTRACT—The distribution of serum albumin is of interest in the *Xenopus (X.) laevis* testis, since albumin is probably a major protein that binds testosterone (T) in the plasma and interstitial fluid. This study was undertaken to determine the localization and purification of serum albumin in the *X. laevis* testis. The interstitial tissue and spermatogonia immunoreacted strongly with a sheep antiserum raised against *X. laevis* albumin. A weak staining was also seen in spermatocytes and early spermatids, but there was no staining in Sertoli cells. In order to clarify whether serum albumin was really localized on the surface of testicular cells in the *X. laevis* testis, a membrane-rich fraction was prepared from testes and extracted with 0.6 M KCl. The KCl extract was then subjected to gel filtration, ammonium sulfate precipitation and high-performance liquid chromatography (HPLC). A protein with Mr=74 kD was obtained by this procedure and its NH_2-terminal amino acid sequence was determined. The sequence of the first 19 amino acids was DTDADXXKXIADVYTALTE, suggesting that this protein was identical to serum albumin (Mr=74 kD). When the membrane fraction of blood cells in this animal was handled in the same manner, no appreciable amount of albumin was detected. These results suggest that the 74 kD serum albumin, possibly associated with bound T, may play an important role in the differentiation of germ cells during spermatogenesis of *X. laevis* testis.

INTRODUCTION

Sperm formation, spermatogenesis, is the result of a complex process of biochemical and morphological differentiation of germ cells. Pituitary gonadotropins and steroid hormones control spermatogenesis [20–22]. As yet, the stage-specificity of hormonal control of spermatogenesis remains unclear. In order to clarify the stage-specificity of steroid hormonal control of this process, immunohistochemical studies have been performed in the mammalian testis using antibodies raised against serum albumin, because this protein has a high capacity to bind T [5] and serves as the major protein transporting T in the plasma and interstitial fluids in adult rats [4]. It is probable that albumin acts on Leydig cells and stimulates steroidogenesis of these cells [5]. In fact, Christensen *et al.* [3] showed under electron-microscopic immunocytochemistry that albumin was localized on the surface of Leydig cells in rat testis, and that immunoreactivity extended between Sertoli cells as well as around spermatogonia and early spermatocytes, but albumin was not present beyond Sertoli cell junctions. In human testis, albumin was observed in Sertoli cells, secondary spermatocytes and early spermatids [6, 15]. The precise localization of albumin within the testis is still controversial.

In amphibians, the regulation of spermatogenesis by steroid hormones is not clear except that T may be required for spermatid formation [17]. This study was undertaken to determine the localization of serum albumin in the *X. laevis* testis, and also to confirm by purifying this protein from the membrane fraction that albumin is really localized on the surface of testicular cells.

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MATERIALS AND METHODS

Experimental animals

Adult male *X. laevis* (50–70 gm) were used for all the experiments.

Immunohistochemistry

Testes were fixed in Bouin's solution and further treated according to conventional histological technique. Sections (approximately 5 μm thick) were cut on a microtome (Yamato), placed on alcohol-washed slides, and warmed on a hot plate for 3 hr and then rehydrated in phosphate buffered saline (PBS; pH 7.4) for 10 min. The avidin-biotin-peroxidase complex (ABC) method [8] was used for immunohistochemical stainings using sheep antisera raised against *X. laevis* albumin (a gift of Dr. D. R. Schöenberg) at a 1: 15000 dilution in PBS.

Purification of a 74 kD protein (albumin)

To confirm whether albumin was really localized on the surface of testicular cells, albumin was purified from the membrane-rich fraction of *X. laevis* testes. The membrane-rich fraction of testes was prepared by the method of Millette *et al.* [12]. Testes were removed, wiped with Kimwipes around the tissue, frozen immediately in liquid nitrogen and stored at −80°C until use. Frozen testes were then thawed and homogenized with a glass-Teflon homogenizer in 40 ml of TBS buffer containing 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KCl in 10 mM Tris-HCl (pH 7.4 at 4°C) [12]. The homogenate was centrifuged at 1000 g for 10 min at 4°C to remove large aggregates and debris. The supernatant was used for preparation of plasma membranes by centrifugation on discontinuous sucrose gradients in TBS. Exactly 2.5 ml of the supernatant was mixed with 2.5 ml of

80% sucrose (w/v) to yield 5 ml of 40% sucrose containing membranes. All the 40% sucrose material (5 ml) was layered on top of 2 ml of 45% sucrose (w/v) in TBS in a cellulose nitrate centrifuge tube (Hitachi RPS 40T). Two ml of 30% sucrose (w/v) in TBS were then layered above the 40% sucrose, followed by 1 ml of TBS to fill the tube. Gradients were centrifuged at 125,000 g for 2 hr at 4°C in a Hitachi SCP 85H2 ultracentrifuge equipped with an RPS 40T rotor. Fractionated material (the interface between 30% and 40% sucrose) was collected, diluted~1: 10 in TBS and pelleted at 125,000 g for 40 min at 4°C. To prepare the membrane-rich fraction of *X. laevis* blood cells the same protocol was used. The membrane-rich fraction of testes or blood cells was suspended in 10 ml of 0.6 M KCl, and stirred for 48 h at 4°C. Then, insoluble materials were removed by centrifugation at 105,000 g for 1 h at 4°C. The resultant KCl extract was fractionated through Sephadex G-200 (Pharmacia) gel filtration. Proteins were eluted with 0.6 M KCl at a flow rate of 10 ml/h. The effluent was collected in 1.8-ml fractions, and the protein content of each fraction was monitored by absorbance at 280 nm. After this, the effluent from the membrane-rich fraction of either testes or blood cells was divided into three fractions. The fraction (designated F2 or F3', respectively) was dialyzed for 12 h at 4°C against 1 liter of saturated ammonium sulfate solution. Precipitates were collected by centrifugation at 105,000 g for 30 min at 4°C and dissolved in 2 ml of 50 mM Tris-HCl (pH 7.4). The sample was applied to a column of Mono Q Sepharose (HR 5/5; Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.4). Protein concentrations were determined by the method of Peterson [16] using bovine serum albumin as the standard.

SDS-PAGE and immunoblot analysis

Proteins were added to the SDS sample buffer, heat denatured, and electrophoresed on a 12% acrylamide gel [10]. For immunoblot analysis, nitrocellulose membranes were stained after transfer [23] with a sheep anti-*X. laevis* albumin serum at a 15,000 dilution in PBS [13].

NH_2-terminal sequence analysis

An NH_2-terminal amino acid sequence analysis was performed using the 74 kD protein obtained from *X. laevis* testes. An automated protein sequence analysis was performed on an Applied Biosystems Model 470A gas-liquid phase protein sequencer connected on-line to an Applied Biosystems Model 120A HPLC [14].

RESULTS

Immunohistochemical studies for localization of albumin

The immunohistochemical localization of serum albumin was examined by use of a highly diluted specific antiserum. None of cells was stained when non-immune serum was used (Fig. 1a). However, a strong staining was observed in the interstitial tissue and spermatogonia, when the sheep antiserum raised against *X. laevis* albumin was used (Fig. 1b). A weak staining was also seen in spermatocytes and early spermatids, but not in Sertoli cells (Fig. 1b).

Purification of a 74 kD protein (albumin)

The membrane-rich fraction from testes was extracted with 0.6 M KCl and then the 0.6 M KCl extract was applied to a Sephadex G-200 column. The effluent was divided into three fractions (Fig. 2a). The last peak was not saved be-

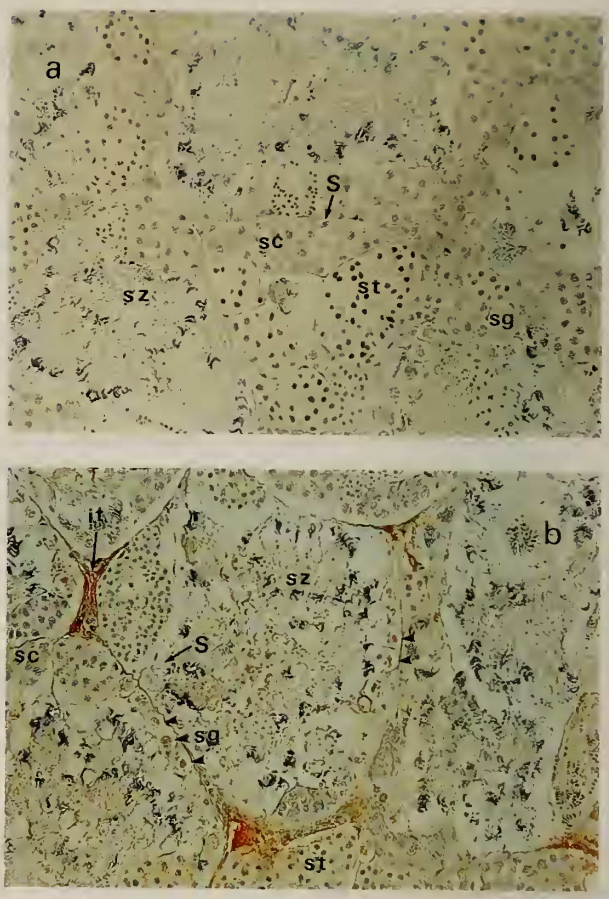


Fig. 1. Localization of albumin in the *X. laevis* testis by indirect ABC analysis with sheep antiserum raised against *X. laevis* albumin.
Immunostaining with sheep non-immune serum (a) and with sheep antiserum raised against *X. laevis* albumin (b). Arrow and arrowheads indicate Sertoli cell and spermatogonia, respectively. Sg, spermatogonia; sc, spermatocytes; st, spermatids; sz, spermatozoa; S, Sertoli cells; it, the interstitial tissue.

cause no detectable amounts of proteins was obtained, although it had an absorbance at 280 nm. This may be due to free amino acids and/or small peptides since all substances in this peak were dialyzable. When the membrane-rich fraction from blood cells was used instead of that from testes, three peaks appeared in the elution profile from the gel filtration (Fig. 2b). The first three fractions were designated F1, F2 and F3 for the testes, or F1', F2' and F3' for the blood cells, respectively (see Figs. 2a and 2b).

The F2 fraction for the testes was dialyzed against a saturated ammonium sulfate solution and then the precipitates were obtained, followed by HPLC. As shown in Figure 3a, a major peak was obtained by the first HPLC. This peak with a dotted area was pooled and dialyzed for 1 hr against 1 liter of 50 mM Tris-HCl (pH 7.4). After dialyzed, the sample was subjected to the second HPLC. When the second HPLC was done, the symmetrical peak with a dotted area containing a 74 kD protein was eluted with 0.25 to 0.35 M NaCl (Fig. 3b). When this peak was analyzed for the

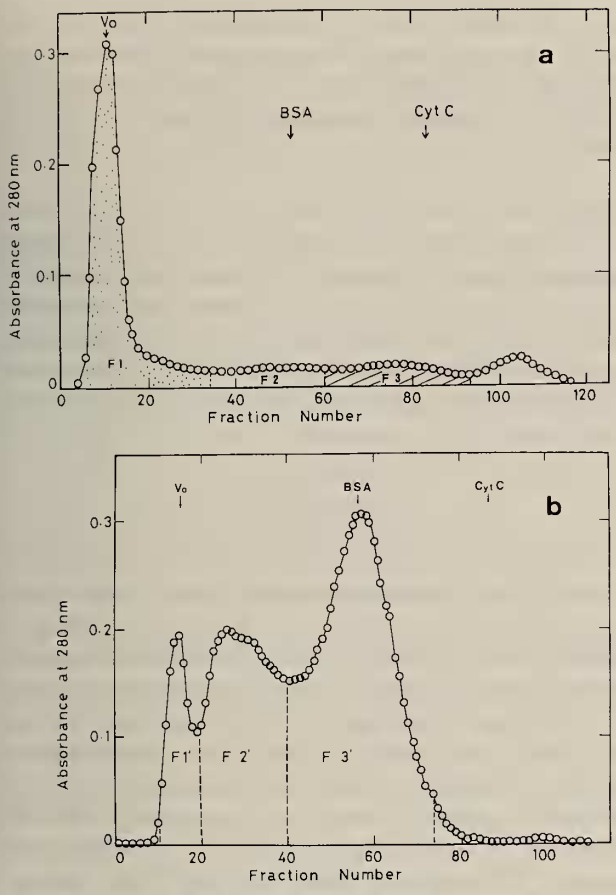
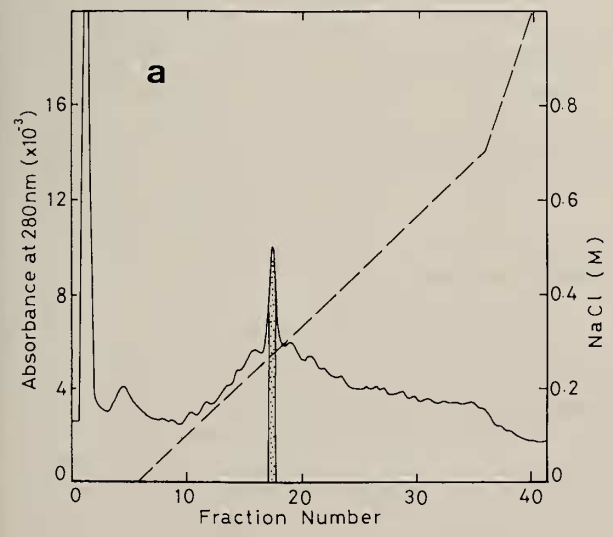


FIG. 2. Gel filtration chromatography on Sephadex G-200 of the 0.6 M KCl extract of *X. laevis* testes (a) and blood cells (b). A column (1.5×120 cm) was calibrated with standard molecular weight proteins [bovine serum albumin (BSA; Sigma, Mr=68 kD)] and cytochrome c (Cyt C; Miles, Mr=14 kD). The void volume (Vo) is indicated with an arrow.

heterogeneity of proteins by SDS-PAGE, a 74 kD protein was not a major protein in the KCl extract (Fig. 4A; lane c). However, the 74 kD protein was a major protein in the precipitate of the fraction F2 obtained by a dialysis against a



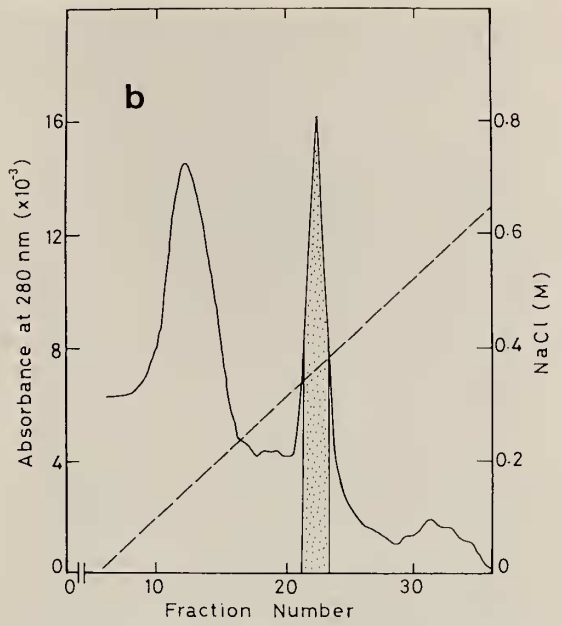
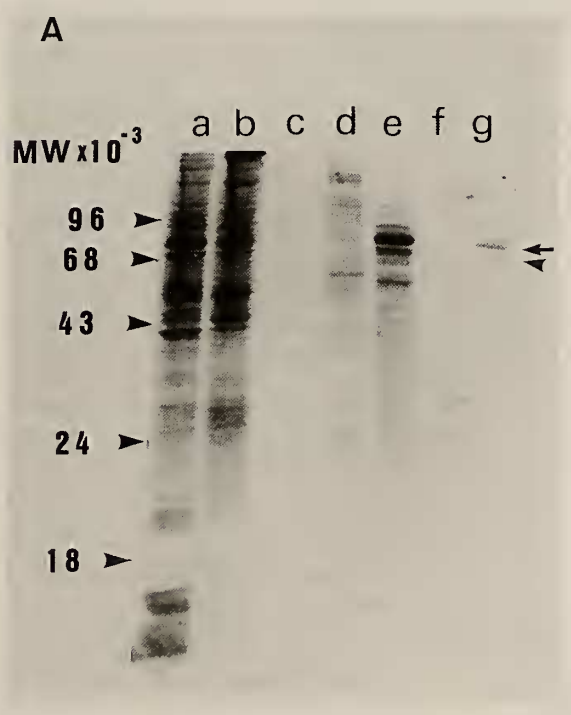


FIG. 3. Elution profiles from the first (a) and second (b) HPLC. Proteins in the F2 fraction obtained from the Sephadex G-200 gel filtration chromatography were eluted with 20 ml of a linear gradient of NaCl (0.0–1.0 M) in 50 mM Tris-HCl (pH 7.4) at a flow rate of 2 ml/min.

saturated ammonium sulfate solution. After the second HPLC, a very strong band with Mr=74 kD and a much weaker band with Mr=68 kD were observed (Fig. 4A; lane g). Based on densitometric tracings of stained gels on SDS-PAGE (Joyce-Loebel Chromatoscan 3), the total amount of the 68 kD protein was <5% of that of the 74 kD protein. In contrast, a 74 kD protein could not be detected



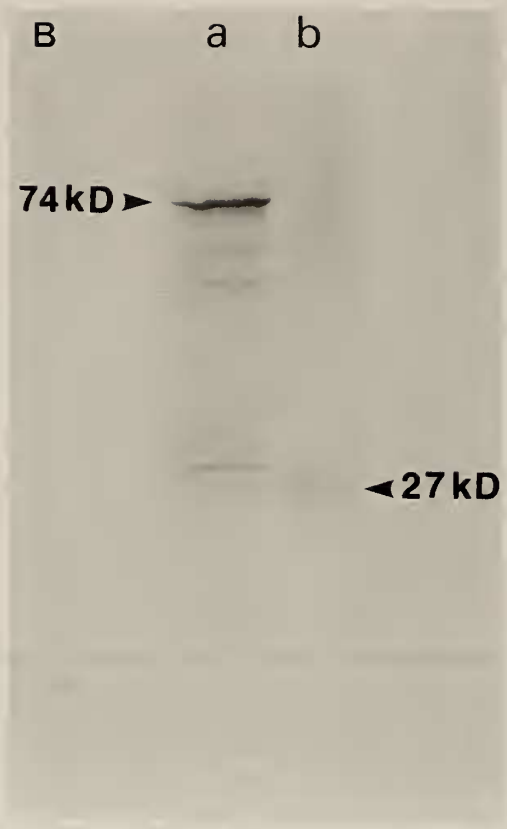


FIG. 4. Profiles of proteins on SDS-PAGE as observed during the purification procedure (A) and the gel filtration (B).
(A): Lane a, the homogenate (50 μg); lane b, the membrane fraction (50 μg); lane c, the KCl extract (5 μg); lanes d-f, the F1 (10 μg), F2 (12 μg), F3 (8 μg) fractions from gel filtration, respectively; lane g, the sample from the second HPLC (2 μg). An arrow and an arrowhead indicate 74 kD and 68 kD proteins, respectively.
(B): Lane a, the precipitate in the F2 fraction from testes after dialysis against a saturated ammonium sulfate solution (10 μg/lane); lane b, the precipitate in the F3' fraction from blood cells after dialysis a saturated ammonium sulfate solution (8 μg/lane).

in the precipitate of the F3' faction from blood cells after dialysis against a saturated ammonium sulfate solution (Fig. 4B; lane b). Yields of each step in the process of purification of the 74 kD protein are summarized in Table 1. The 74 kD protein was obtained to a final yield of 0.1%.

Identification of the 74 kD protein

In order to identify the 74 kD protein, an NH_2-terminal amino acid sequence analysis of this protein was performed. As seen in Fig. 5, the sequence of the first 19 NH_2-terminal amino acids of the 74 kD protein was identical, except for 3 unidentified amino acids, to that of the 74 kD *X. laevis* serum albumin published by Maskaitis et al. [11] and Schorpp *et al.* [18].

DISCUSSION

This study has clearly shown that serum albumin is present in the interstitial tissue of the *X. laevis* testis. According to Christensen *et al.* [3], immunoreactivity of albumin was detected on the surface, but not in the cytoplasm of Leydig cells. It is not clear in this study whether both the cell surface and cytoplasm of cells in the interstitial tissue of the *X. laevis* testis contain albumin. We need further investigation at an ultrastructural immunocytochemical level to answer this question. However, it seems probable that the surface of cells in the interstitial tissue [probably steroid hormone (SH)-secreting cells] is associated with albumin, since albumin was purified from the membrane-rich fraction of *X. laevis* testes, but not from membranes of blood cells. Spermatogonia also had a strong response to the albumin antibody, and spermatocytes and early spermatids had a weak response. A question also arises as to whether albumin is localized on the surface of these germ cells. Presently, we have no direct evidence for this. Immunocytochemical stu-

TABLE 1. Yields of the 74 kD protein from *X. laevis* testes.

	Total protein (mg)			Yield (%)
	exp. 1	exp. 2	exp. 3	
Membrane fraction	23.0	28.0	37.8	100
KCl extract	3.63	4.59	7.23	17.4
Sephadex G-200 gel filtration	0.240	0.320	0.425	1.11
HPLC	0.025	0.024	0.038	0.10

FIG. 5. The N-terminal sequence of the 74 kD protein from the *X. laevis* testis.

	Prepeptide	Propeptide	Mature Protein
74kD Albumin[a]	MKWITLICLLISSSFIES	RILFKR	DTDADHHKHIADVYTALTERTFKG·····
This work			******XX*X****************

[a]Data from Schorpp *et al.* [18]. The leader peptide of the 74 kD *X. laevis* albumin consists of a hydrophobic sequence of 24 amino acids [11,18].
Note. Regions of identity are noted by an asterisk. X, not determined.

dies will answer this question.

It is of great interest to note that Sertoli cells did not respond to the antibody. Several investigators have localized albumin in Sertoli cells of mammalian testes such as human [6, 15], hamster [9] and rat [3]. It is not clear presently why the immunoreactivity of albumin was not observed in Sertoli cells of *X. laevis* testis. In the seminiferous tubules of mammalian testis, Sertoli cells form a barrier, so-called the blood testis-barrier, to retard or exclude many substances in the blood plasma from entrance into the lumen [19, 25]. Most germ cells, except for spermatogonia, reside within the barrier or the adluminal compartment. In anurans, on the other hand, spermatogenesis takes its course in the cysts of the testes. Germ cells develop within groups of "follicle" cells which are thought to be comparable to the Sertoli cells in the mammalian testis. According to Bergmann *et al.* [1], substances like nutrients and hormones in the blood in this species is probably accessible to most developing germ cells. Taking all these findings into consideration, it is not surprising that Sertoli cells had no response to the antibody of albumin. Perhaps, albumin is not associated with Sertoli cells. T may be transported to germ cells from the interstitial space without going via Sertoli cells.

Finally, the HPLC sample consisted of two proteins, as judged from the result of SDS-PAGE analysis. One with Mr=74 kD was a very strong band and the other with Mr=68 kD was a very faint band (see Fig. 4A; lane g). Both bands immunoreacted with the antibody of albumin (data not shown). This is not unusual. The frog, *X. laevis*, has two albumin genes that code for a 74 kD and a 68 kD serum albumin [11, 18]. In addition, two molecular forms of proteasome [7], calreticulin (a Ca^{2+}-binding protein) [24] and prolactin [26] have also been reported in this animal. Two forms of these proteins may have occurred from a duplication of the entire genome in the genus *Xenopus* [2]. In view of these findings, we must have purified two albumins together, but could not separate one from another by the methods used in this study. One explanation for this may be as follows; albumin exists in two forms that migrate on SDS-PAGE with relative molecular weights of 74 kD and 68 kD, respectively. As the number of amino acids of the two albumins is equivalent (608 residues), the anomalous behaviour on SDS-PAGE may be due to the glycosylation, which is specific for the 74 kD albumin [18]. It might be possible to separate one from another by changing the range of NaCl concentrations on HPLC.

As to which albumins are more closely associated with immunoreacted cells remains unclear at the present time. In the serum of *X. laevis*, the 74 kD albumin exists to a much greater extent than the 68 kD albumin (data not shown). It seems, therefore, very likely that the former is more closely associated with the surface of testicular cells. We do not know yet how spermatogenesis in *X. laevis* is controlled by T. Nevertheless, it is extremely interesting to note that the developing germ cells and the interstitial tissue (probably SH-secreting cells) are associated with albumin. Considering that serum albumin can bind T, spermatogenesis may be influenced under T with the aid of serum albumin in this species as well as in others.

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